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Fourth quarterly report on Contract #W-49-057-cws-37

Covering the period 10/1/47 to 12/31/47

The investigations which followed the discovery of di-isopropyl fluorophosphate (DFP) confronted the scientists with two distinct problems:
(i) whether or not cholinesterase activity is essential for conduction,
(ii) whether or not the high toxicity of this compound must be attributed exclusively to the reaction with cholinesterase.

1. NECESSITY OF CHOLINESTERASE FOR CONDUCTION

It has been shown in many ways that conduction cannot be dissociated from cholinesterase activity. Reexamination of earlier work reporting the possibility of conduction in the bullfrog sciatic in the complete absence of cholinesterase has shown that the manometric method used for the determination of cholinesterase activity was in this particular case inadequate due to several adverse factors. When the acetylcholine hydrolysis was determined with the frog rectus abdominis method, it was found that although more than 90 percent of the initial enzyme concentration was inactivated, about 7 to 8 percent still persisted.

Recently, experiments have been described which in the opinion of the investigators, show that conduction can continue in nerves exposed to DFP in the complete absence of cholinesterase (Boyarski, Tobias, & Gerard). The acetylcholine hydrolysis by cholinesterase was determined with the frog rectus abdominis technique. In these experiments, the DFP was dissolved in peanut oil and for low DFP concentration (0.003M) the nerves were immersed in the oil for a considerable length of time (3 hrs.), without effect on the action potential. No acetylcholine hydrolysis was found. Since DFP has a high lipoid solubility, the rate of penetration could be expected to be smaller than if the nerves were exposed to DFP in aqueous solution. The investigators apparently thought it possible, with this milder form of exposure, to obtain the inactivation of the enzyme without "toxic" effects held to be responsible for the abolition of conduction.

We have repeated these experiments under the same experimental conditions. For the determination of the acetylcholine hydrolysis by the remaining cholinesterase, however, two essential changes were made. Since on the basis of our previous experiments, it could be expected that 100 mg. of nerve could split 20 to 30 µg. per hr., the low amounts of tissue and the high initial amount of acetylcholine used in the experiments of Boyarski, et al., made it a priori improbable that the method could indicate the remaining activity. A more appropriate enzyme to substrate ratio was therefore chosen for our determinations. The solution of acetylcholine to which the suspension of the experimental nerve was added contained initially only 150 µg. instead of 420 µg. and the amounts of nerve used were 120 to 140 mg. instead of 20 to 50 mg.

One of the experiments carried out is described in detail. Seven frog sciatic nerves weighing 186 mg. were exposed to DFP in peanut oil (0.003M) for 3 hrs. As reported by the previous workers, the action potentials were unimpaired. The nerves were then washed in Ringer's solution for 1 hr. and ground in 2.0 cc. of 0.01M phosphate buffer. 1.5 cc. of the suspension containing 139.5 mg. of nerve tissue was put into a vessel to which was added 0.2 cc. acetylcholine solution containing 150 µg.

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of acetylcholine. This was incubated for 3 hrs. at room temperature. Another vessel used as control contained the acetylcholine solution and phosphate buffer, but no nerve tissue, and was treated in exactly the same way. At the end of the incubation period, 0.5 cc. were taken from the control and the experimental vessels and added to 2.5 cc. of 0.01M phosphate buffer. The deproteinization was carried out in the usual way, 0.8 cc. of 0.1M phosphate buffer and 0.25 cc. of n/HCl were added to both samples and left for 30 min.; then 0.25 cc. n/NaOH was added for neutralization. The samples were centrifuged and the supernatant was assayed for acetylcholine with the frog rectus abdominis as described previously.

Fig. 1 shows the assay. Although the control was diluted 100 times before the assay, 0.31 μg. of acetylcholine were found in 3 cc. used for the determination. The solution exposed to the nerves suspension was diluted only 50 times, but nevertheless, 0.15 μg. of acetylcholine were found in 3 cc. Calculated per vessel, the amount of acetylcholine in the control was found to be 151 μg. In the experimental vessel it was only 37 μg. Consequently, the suspension containing 139.5 mg. of nerve had split 114 μg. of acetylcholine in 3 hrs. Calculated per g. nerve per hr., the hydrolysis of acetylcholine amounts to 272 μg. after the exposure to the critical concentration of DFP for 3 hrs.

In the report mentioned, the investigators were unable to detect any DFP retained in the exposed nerves. In all cases tested in the usual way, we found the usual retention amounting to about 30 to 40 percent in agreement with previous findings. In the particular experiment described above, the retention was found to be 40 percent. Corrected for inhibition due to the retained DFP, the cholinesterase present in the nerves thus exposed to DFP was actually capable of splitting acetylcholine at a rate of 453 μg. per g. per hr.

2. KINETIC ASPECTS OF CHOLINESTERASE INHIBITION TO DFP

DFP is one of the most specific and most powerful enzyme inhibitors known. Although its toxicity must be referred to this single chemical reaction, the investigations mentioned have demonstrated that the effectiveness of a drug depends on a great variety of factors. Some of these factors were found in experiments on isolated nerves or on the whole animal, others, in *in vitro* experiments.

Some kinetic aspects of the enzyme inhibition of DFP have now been studied in order to determine whether or not additional factors have to be considered in the interpretation of the mechanism of DFP action. Such an analysis has been made possible by the availability of a virtually pure cholinesterase preparation, showing only one component in the analytical ultracentrifuge run.

Cholinesterase was prepared by fractional ammonium sulfate precipitation from the electric tissue of *Electrophorus electricus* as recently described. About 50 cc. of a solution was obtained, capable of splitting 2500 gm. of acetylcholine per hr. One mg. of protein could hydrolyze 20,000 mg. of acetylcholine per hr. The enzyme solution was dialyzed against distilled water for 9 days, then sub-divided into five equal portions and lyophilized. A fine white powder was obtained and kept for several months in the refrigerator in sealed ampoules. One tube of lyophilized cholinesterase was dissolved in 30 cc. of solution containing the following salts: 0.1M NaCl, 0.01M $MgCl_2$, 0.015M phosphate buffer pH 7.4. The solution could hydrolyze approximately 500 gm. of acetylcholine per hr. which indicated that the process of lyophilization and storage of the powder for several months in the icebox had no effect on the activity of the enzyme.

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The enzyme solution was centrifuged at 36,000 R.P.M. for 1 hr. at about 9°C. The pellet was redissolved in 4 cc. of phosphate buffer described above. Since there were some undissolved particles, the solution was centrifuged and the residue washed twice with 3 cc. of buffer. One cc. was capable of splitting 24.4 gm. of acetylcholine per hr. More than ½ of the enzyme activity was lost by the procedure. We are unable to account for this loss since in previous experiments, ultracentrifugation did not affect the activity under similar conditions. In the solution obtained, 1 mg. of protein was capable of hydrolyzing about 60,000 mg. of acetylcholine per hr., which is close to the highest degree of purity obtained previously. This enzyme solution has been used in all experiments described.

The incubation at 10°C and the manometric determinations of the cholinesterase activity at this temperature were carried out in a refrigerated Warburg bath.

Optimal incubation period -- For the study of the kinetics of the inhibition of an enzyme, the possibility of working with a wide range of enzyme concentration is a great advantage. Rather high enzyme concentrations appeared advisable for determining whether or not the effect of DFP on the enzyme occurs on a mole-to-mole basis. The enzyme concentration of the preparation available was 1.4×10^{-7}M calculated on the assumption of a molecular weight of about 3,000,000. This figure is based on the sedimentation rate in the analytical ultracentrifuge run. The concentration appeared to be proper for the investigations planned.

At first, an incubation period had to be found which offered the optimal condition. Since the inhibition of the enzyme is progressively irreversible, a prolonged incubation period would lead to the destruction of the greatest part of the enzyme, whereas the measurement of the effectiveness during a short incubation period might be too small for accurate estimation. To 0.5 cc. of the enzyme solution, 0.5 cc. of a solution was added of which the DFP concentration was 3×10^{-6}M. The degree of inhibition was determined at varying periods of incubation by removal of an aliquot part of the solution and dilution to 5,000 times its original volume. By this dilution, the concentration of DFP falls far below its inhibitory range and that fraction of the enzyme which had not been irreversibly inactivated could be determined manometrically. As may be seen from the data of Fig. 2, at 23°C, inactivation of about 50 percent is obtained after an incubation period of 150 min. During this period, the percentage of inactivation rises at a rather high rate, whereas later, the increase progresses more slowly. After 300 min. incubation, the fraction of enzyme inactivated has risen to 66 percent.

The experiments were repeated at 10°C. The percentage inhibition as may be seen from the data of Fig. 2 was slightly smaller than at 23°C. The shape of the curve was however about the same. After 150 min. of incubation, 39 percent of the enzyme were inactivated, after 300 min. about 55 percent. At first glance, it may seem surprising that the difference between 10 and 23°C is so small. But obviously, two antagonistic factors are involved: at 23°C, the rate of hydrolysis by active enzyme will be higher; on the other hand, the rate of inactivation of cholinesterase by DFP will also be increased at higher temperature and therefore less active enzyme will be left at the end of the incubation period. These antagonistic effects may account for the rather small difference observed.

From the data obtained, an incubation period of 150 min. at a temperature of 10°C appeared to be a favorable condition for the study of the varying factors involved and has been used for the following observations.

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Relation between enzyme and inhibitor concentrations -- Having thus established the optimal incubation period, the concentration of the inhibitor during the incubation was varied in order to test whether or not the effect changed proportionally. As may be seen from Fig. 3, the inhibitory effect of DFP at a concentration lower than that required for 50 percent inhibition at 150 min. incubation, decreased strictly proportionally. This suggests an inactivation of the enzyme by the inhibitor on a stoichiometric basis. The data obtained in a series of experiments were so close to each other that only one set is reproduced in the Figure. With twice the concentration required to inactivate 50 percent of the enzyme, the percentage inhibition rose to 86.5 percent. This is not strictly proportional, but may have been expected in that high range of inhibition.

Excess of inhibitor over enzyme concentration -- Although the strict proportionality between increase of inhibitory effect and the increased concentration of the inhibitor suggested an action on a mole-to-mole basis, it appeared surprising that the concentration of the inhibitor, under the experimental condition used, was 25 times as high as the concentration of the enzyme.

In order to find out the role of the enzyme concentration in this connection, a series of tests were made varying the enzyme concentration from 10^{-7} to 10^{-11}M. As may be seen from Fig. 4, it was found that the excess required increases rapidly with dilution. If pE, the negative log of the molar concentration of the enzyme is plotted against the log of the inhibitor concentration $[I]$ over the enzyme concentration, $[E]$, a straight line is obtained. Whereas the excess of molecules of inhibitor over molecules of enzyme is 25 in the highest enzyme concentration used, more than 100,000 molecules of inhibitor are necessary for each molecule of enzyme in the lowest concentration tested in order to obtain the 50 percent inactivation.

It appears likely that the line of Fig. 4 remains straight at higher concentrations than those tested. In that case, extrapolation to the point log $\frac{[I]}{[E]}$ - 1 would give a value of approximately 6. At this concentration, one cc. of enzyme would hydrolyze between 350 and 400 gm. of acetylcholine per hr. Such a concentration of the enzyme may be obtained. In earlier experiments, an enzyme solution was prepared, one cc. of which was capable of hydrolyzing 500 gm. of acetylcholine per hr. Whether such a concentration may occur in nature, is at present, difficult to decide. The highest activities found for electric tissue of the eel and with the head ganglion of Squid, corresponded to a hydrolytic power of 6 to 7 gm. of acetylcholine per gm. fresh tissue per hr. Since it is known that the enzyme is concentrated exclusively in the neuronal surface, it is possible that if the active membrane is a layer only a few molecules thick, the concentration there may be of this order of magnitude or even higher. Independent of this physiological problem, it would be of interest for the study of the kinetics of the enzyme inhibition, to test the inhibitory effect in such extreme concentrations. However, for the time being, no such preparation is on hand and the experiment must be postponed for later studies.

Difference between DFP, eserine and prostigmine -- The fundamental difference of the chemical reaction between cholinesterase and DFP as compared with that of other known inhibitors is obviously the progressive irreversibility. It appeared worthwhile, however, to investigate whether or not there are additional variables on which the effect of these two types of inhibitors may depend.

Since the reaction between prostigmine and cholinesterase is totally reversible, an incubation with a high enzyme concentration and subsequent dilution of several thousand times as has been used in the case of DFP, should lead to complete inactivation and therefore, have no effect. This has been checked and confirmed. In experiments with diluted solutions, the immediate effect of prostigmine and eserine is in striking contrast to the slowly increasing action of DFP. As may be seen in Fig. 5, incubation with eserine for varying periods of time, up to 150 min., does not alter the degree of inhibition. The same has been found with prostigmine. The percentage of inhibition produced by DFP rises continuously. In this case, the inhibition represents the total effect, the reversible as well as the irreversible part, since at the end of the incubation period, the enzyme activity has been tested without dilution. Whereas, for short periods of contact, prostigmine and eserine are, at the same concentration, stronger inhibitors than DFP, the effect becomes equal to that produced by DFP after 150 min. incubation and will be stronger than that of the two alkaloids if the enzyme remains in contact with the inhibitor for still longer periods of time. It is obvious that this factor will influence the toxicity in vivo. In warm-blooded animals, i.e., at 37°C, the reaction rate between DFP and enzyme will be much higher than that observed at the low temperature used in these experiments. But for short periods of time, a considerable difference may be expected between the two types of inhibitors.

In another respect, the experiments have revealed a significant difference. In low concentrations, at 10^{-6}M to 10^{-7}M, prostigmine and eserine have a much stronger effect than DFP, even after 150 min. incubation. However, with increasing concentration, the effectiveness of DFP increases markedly and at about 3×10^{-5}M, it surpasses that of the two alkaloids. Fig. 6 shows the inhibitory effects obtained by varying the inhibitor concentration. These data were obtained with a constant incubation period of 150 min. at 10°C. Since the latter factor has no significant effect on the inhibitory action of the alkaloids, its change would influence only the DFP curve. At a given concentration, therefore, significant differences in effect may be produced by merely altering the incubation time.

The experiments offer additional support for the conclusion that the great variety of toxic symptoms by DFP poisoning must not be attributed to a multitude of chemical reactions but to the great number of variables influencing the course of the single reaction with cholinesterase.

LEGEND OF FIGURES

Fig. 1. Test of cholinesterase activity in a frog sciatic nerve after exposure for 3 hrs., to DFP in 0.003M concentration (dissolved in peanut oil). The ACh hydrolysis is determined by bioassay with the frog rectus abdominis. ACh is added to the homogenized nerve suspension and the amount of the ester present after 3 hrs. incubation is tested. The initial amount of ACh is determined in a control vessel treated in the same way but without addition of nerve tissue. Below the tracings of the response to the standard solution is given the amount of ACh in μg. applied to the muscle in 3 cc. of frog Ringer's solution. The content of the experimental vessel (E) was diluted 50 times after deproteinization; that of the control vessel (C) 100 times. In spite of the 2-fold greater dilution, the control vessel contains more than twice the amount of ACh present in the experimental vessel. The total amount of ACh in the experimental vessel, on the basis of this bioassay, is 37 μg; in the control vessel, 151 μg. Consequently, 114 μg. were hydrolyzed.

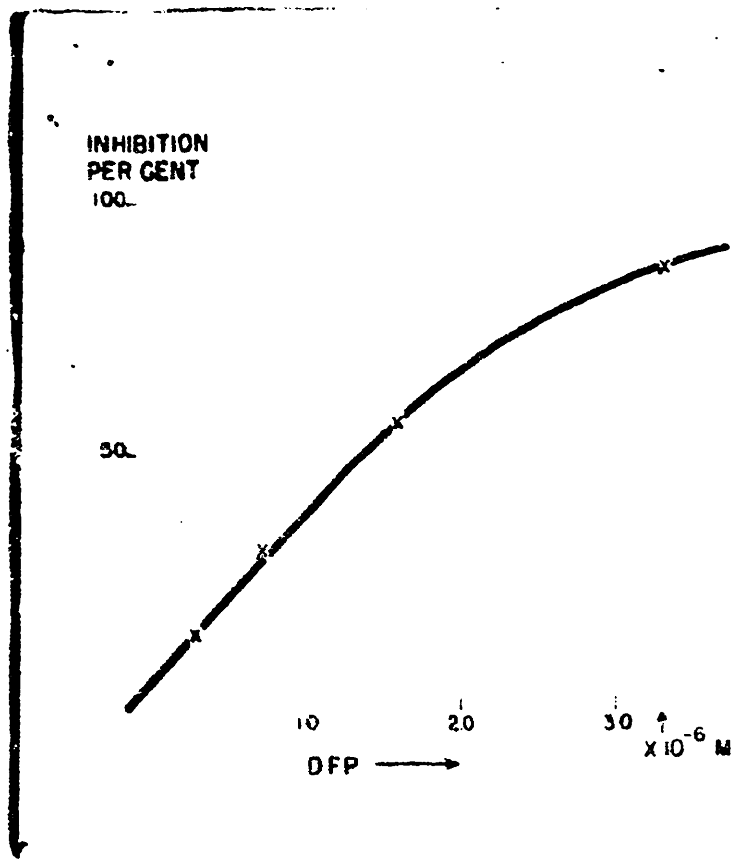
Fig. 2. Inactivation of cholinesterase by DFP at varying periods of incubation. The DFP concentration used was 1.5×10^{-6}M. The enzyme concentration about 7×10^{-8}M. x ------- x 23°C o------o 10°C.

Fig. 3. Relationship between the percentage of enzyme inhibition and the concentration of DFP. Up to 50 percent, the inhibition increases strictly proportionally to the increase of DFP concentration suggesting a reaction on a mole-to-mole basis.

Fig. 4. Excess of DFP required for varying enzyme concentrations. pE, the negative log of the molar concentration of the enzyme is plotted against the log of the inhibitor concentration [I] over the enzyme concentration [E].

Fig. 5. Difference between the effect of incubation on cholinesterase inhibition by DFP and by eserine. The prostigmine effect, like that of eserine, is unaffected by incubation. The DFP concentration as well as that of eserine was 1×10^{-6}M.

Fig. 6. Effectiveness of inhibition of cholinesterase by DFP and the alkaloids prostigmine and eserine at varying inhibitory concentrations. Inhibition in percent plotted against pI, the negative log and the molar concentration of the inhibitors. In the experiments with DFP, the enzyme was incubated for 150 min., with the inhibitor before the determination. No incubation time was used in the experiments with the alkaloids.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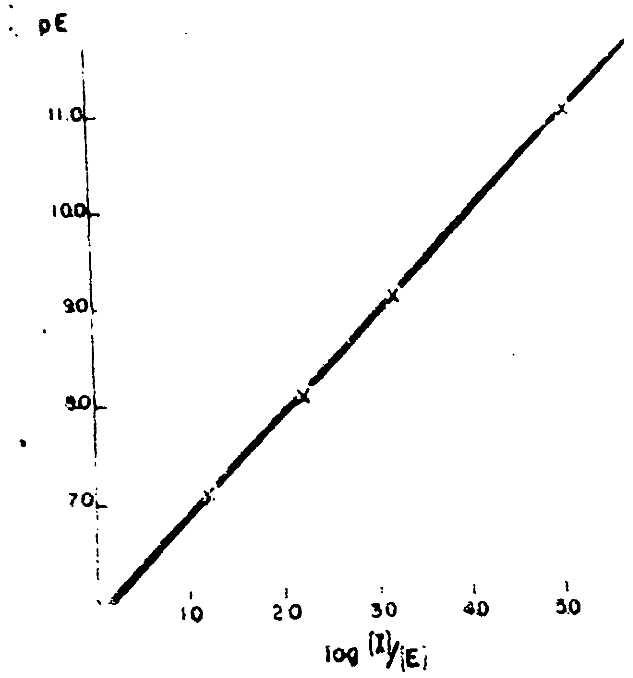

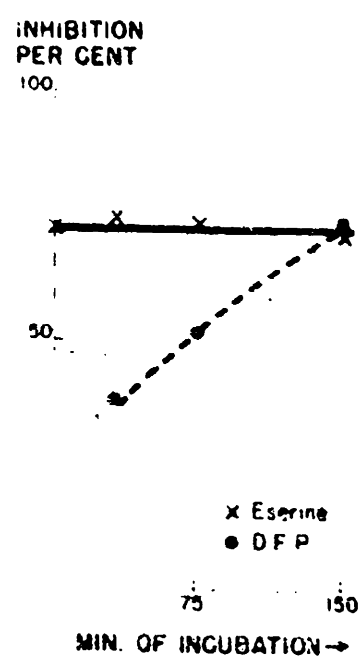
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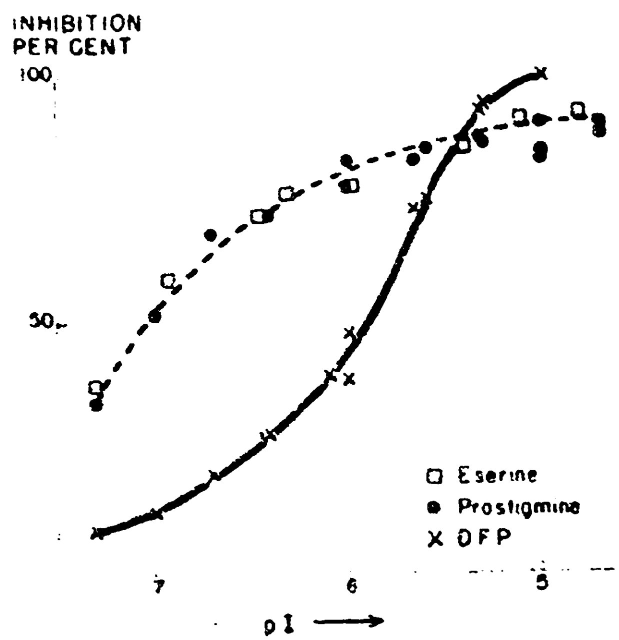

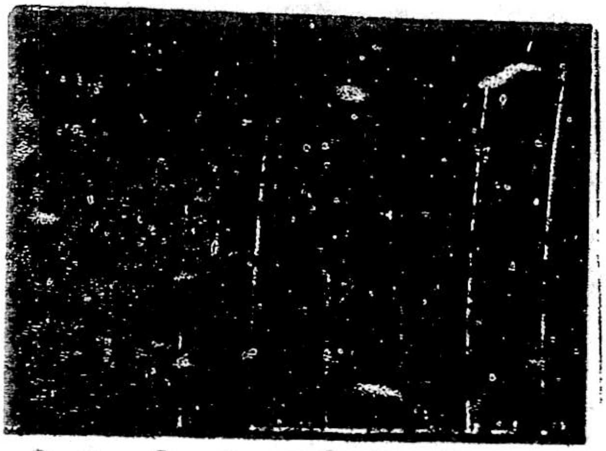

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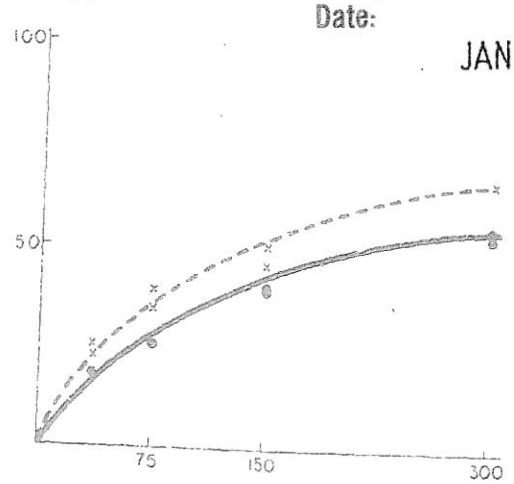

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DEPARTMENT OF THE ARMY
US ARMY RESEARCH, DEVELOPMENT AND ENGINEERING COMMAND
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RDCB-DPS-RS

28 NOV 2011

MEMORANDUM THRU Acting Technical Director (RDCB-D/Mr. Alvin D. Thornton), Edgewood Chemical Biological Center (ECBC), 5183 Blackhawk Road, Aberdeen Proving Ground, MD 21010-5424

FOR Department of Defense, Washington Headquarters Services, 1155 Defense Pentagon, Washington, DC 20301-1155

SUBJECT: OSD MDR Case 11-M-1022 (DTIC Case No. ADC955165)

1. In August 2011, ECBC received a request for a "Freedom of Information Act (FOIA) like review" of documents dealing with Di-isoprophl Fluorophosphate (DFP) provided by the Department of Defense, Washington Headquarters Services (OSD MDR Case 11-M-1022), regarding Defense Technical Information Center (DTIC) Case No. ADC955165.

2. The aforementioned DTIC documents were reviewed by Subject Matter Experts from ECBC on Aberdeen Proving Ground, Maryland. All pages are approved for declassification and can be made available for release to the public.

3. The point of contact is Mr. Ronald L. Stafford, the ECBC Information Security Officer, (410) 436-6810 or ronald.l.stafford.civ@mail.mil.

JUNE K. SELLERS
Security Manager

11-M-1022